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An Assessment of Transformation Protocols of Trametes versicolor: A Possible Biological Pretreatment for Biomass

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An assessment of transformation protocols of *Trametes versicolor:* a possible biological pretreatment for biomass

by

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Table of Contents

Abstract

Lignin is an obstacle in large-scale biofuel production. This can be overcome by the use of fungal pretreatment. This experiment assessed two transformation assays, based on the protocols of *Laccaria bicolor* and *Phanerochaete chrysosporium*, and applied them to *Trametes versicolor*. In order to determine a successful transformation, *T. versicolor* was transformed with green fluorescence protein (GFP) using the two assays, followed by detection of transformed mycelia under UV light. The two protocols were assessed based on timeliness, ease, and effectiveness. Based on the quantity of viable transformants and ease, the membrane assay described for *Laccaria* is successful when the antibiotic concentration, specifically, is optimized and applied to *T. versicolor*. This work is also the first to show that the plant-based promoter, cauliflower mosaic virus (CaMV) 35S, is effective for expression of any gene in *T. versicolor*, a white-rot fungus.

Introduction

The dependence of human society on energy is a concern because of the increasing population size and energy consumption, and the decreasing availability of fossil fuel sources. Biofuels are an alternative to typical energy sources, and are a renewable source of energy that can theoretically meet our current energy demands (Hill et al., 2006). One potential candidate for successful biofuel sources is plant biomass because it is renewable, inexpensive, and readily available. For instance, plant biomass has been successfully used in the production of biodiesel and bioethanol (Margeot et al., 2009). In order to maximize the output of biofuel sources, pretreatments are applied to enhance the efficiency of downstream processing (Himmel et al., 2007; Kumar et al., 2009). Pretreatments are often costly and chemical-based; however, a substitute is fugal pretreatment because it is economical and safer than traditional methods (Canam et al., 2013).

White-rot fungus has the unique capacity to degrade a wide variety of structurally diverse organic compounds including persistent lignin, cellulose, and hemicellulose (Bumpus and Aust, 1987). Lignin is found in higher plants and is a major component of cell walls. It is also the substance that joins contiguous cells and forms the middle lamella (Kirk and Farrell, 1987). Wood and other vascular tissues are 20-30% lignin, which makes it one of the most abundant renewable materials known (Kirk and Farrell, 1987).

Lignin is a formidable obstacle when attempting to utilize biomass for bioethanol production on a large scale. The white rot classification of fungi, such as *Trametes versicolor* and *Phanerochaete chrysosporium,* possess greater delignification ability than brown-rot and soft-rot fungi (Collins and Dobson, 1997; Martinez et al., 2009). To expedite and increase the lignin degradation, white-rot fungi have been used as pretreatment for biomass to ensure the

maximum availability of organic sugars (Canam et al., 2011; 2013). White-rot fungi can produce unique oxidizing chemicals, including hydroxyl radicals, and produce a suite of lignin-degrading enzymes, such as manganese peroxidase and laccase, which are capable of cleaving bonds found in the lignin compound (MacDonald et al., 2011). Previous studies were successful in reducing the cellulose to lignin ratio when applying a white-rot fungus pretreatment, therefore increasing the ability of biocellulose nearly four times in some cases (Dias et al., 2010; Canam et al., 2011).

Trametes versicolor, commonly referred to as 'turkey tail fungus', is a well-studied white-rot fungus that is native to North America. *Trametes* secretes enzymes, such as phenol oxidase, laccase and lignin peroxidases, which are essential enzymes in the lignin degradation process (Collins and Dobson, 1997; Jonsson et al., 1998). This fungal genome has also been sequenced, which makes it a prime candidate for the creation of new transgenic lines. Canam et al. (2011) examined a mutant strain of *Trametes*; m4D that is deficient in cellobiose dehydrogenase (CDH). This mutant strain was unable to consume glucose, yet it retained all of its lignin degrading capacity. This CDH-deficient mutant strain improved the saccharification of canola straw, enhanced lignin removal, and impaired cellulose catabolism (Canam et al., 2011). Therefore, altered strains of *T. versicolor* are possible fungal pretreatment candidates.

Transforming fungi using *Agrobacterium*-mediated transformation is often the route used in the production of transgenic strains. *Agrobacterium tumefaciens,* a pararetrovirus, is a plant pathogen which induces crown gall tumors in plants (Stafford, 2000; Michielse et al., 2005). When *A. tumefaciens* is introduced to the host, it is able to incorporate its own DNA into the host's genome, where it is later expressed (Figure 1). *Agrobacterium*-mediated transformations yield high percentages of stable transformants with single-copy integrated DNA (Michielse et al., 2005). *Agrobacterium* is successful in its natural plant host range; however, this style of

transformation has also been successful in fungi such as *Lacccaria* and *Phanerochaete*, with slight modifications to the standard plant transformation assays (de Groot et al., 1998; Michielse et al., 2005).

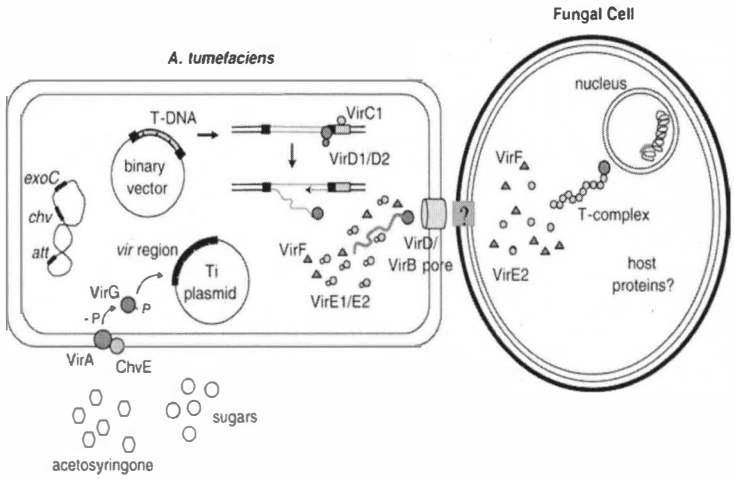

Figure 1: Mode of incorporation of *A.tumefaciens* DNA into a fungal host (Michielse et al., 2005).

Green fluorescent protein (GFP) is a common marker used in transformation protocols. GFP is a gene acquired from the *Aequorea victoria* jellyfish that fluoresces under ultraviolet light, although it typically has an excitation wavelength of 395 nm and emission wavelength of 508 nm (Lorang et al., 2001). GFP is a preferred marker because its fluorescence requires no other co-factor, the tag does not alter the normal function or localization of the fusion partner, and its incorporation is easily detected without having to extract nucleic acids or proteins to confirm successful transformation (Chalfie and Kain, 2006). GFP has been successfully inserted into plant, animal, and fungal cells (Haseloff et al., 1997; Chalfie and Kain, 2006).

Transforming white-rot fungus, particularly *T. versicolor*, has been accomplished through difficult and time-consuming protoplast transformation techniques (Addleman and Archibald,

1993; Dumonceaux et al., 2001). We believe that *Agrobacterium*-mediated transformation can be an effective, alternative technique for *T. versicolor*. Herein, we assess two previously successful transformation protocols that were effective for *Laccaria bicolor* (Kemppainen and Pardo, 2011) and *Phanerochaete chrysosporium* (Sharma and Kuhad, 2010), and applied them to *T. versicolor*. A plant-based binary plasmid will be used to express GFP as well as antibiotic resistance markers in *Trametes,* as a means to detect successful transformation events. The markers are expressed under the cauliflower mosaic virus (CaMV) 35S promoter (Benfey et al., 1989) from a pararetrovirus that infects plants and replicates by reverse transcription, but does not integrate its genome into the host (Shababi et al., 2006). Once a suitable *Agrobacterium*-mediated transformation protocol is determined, it will be used in subsequent experiments focused on the production of future mutant *T. versicolor* for the treatment of potential biofuel feedstocks, such as hybrid poplar, switchgrass, and *Miscanthus* (Somerville et al., 2010).

Method and Materials

Construction of pGFP

A plant expression vector (p35S MYC; Tsai et al., 2012) was constructed by amplifying the cauliflower mosaic virus (CaMV) 35S promoter from pCAMBIA2201 (CAMBIA, Canberra, Australia) and ligating a *Hin*dIII-digested fragment to pCAMBIA1390 (CAMBIA) using the In-Fusion Advantage PCR Cloning Kit (Clontech, Mountain View, CA, USA). The NOS terminator from pCAMBIA1302 (CAMBIA) was similarly cloned as an *Eco*RI fragment downstream of the 35S promoter. pGFP (Figure 2) was derived from p35S MYC by amplifying a green fluorescent protein (GFP; *mgfp5*) from pCAMBIA1302 (CAMBIA) and inserting as a *Bam*HI fragment downstream of the 35S promoter using In-Fusion technology. The plasmid is designed to allow incorporation of the region between the left border (LB) and right border (RB) into the host organism (Figure 2), which should then express GFP and an enzyme that confers resistance to the antibiotic hygromycin.

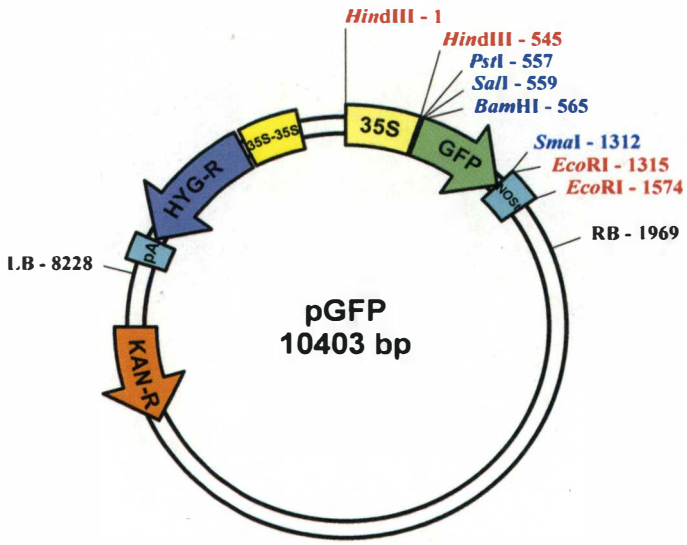

Figure 2: pGFP plasmid containing the gene for green fluorescent protein (GFP) that was incorporated into *Trametes versicolor*.

Production of Competent Agrobacterium cells

Competent *Agrobacterium* cells were grown in 5 ml of YEP medium (10% peptone, 10% yeast extract, 5% sodium chloride) at 28°C and 250 rpm overnight. From the overnight culture 2ml were added to 50 ml YEP in a 250 ml flask, and the new suspension was incubated at 28°C and 250 rpm until the optical density (at 600 nm) measured 0.5-1.0. The culture was set on ice and then centrifuged at 8,000 rpm of 10 min at 4°C. The supernatant was discarded and the cells were resuspended in 1 ml of filter-sterilized 20 mM $CaCl_2$.

The competent cells were incubated on ice for 5-30 min and subsequently heat-shocked by placing them in a 37°C water bath for 5 min. One milliliter of YEP medium was added and the cells were then incubated at 28°C for 2 h. After the recovery period, the cells were centrifuged at maximum speed for 1 min and the supernatant was discarded. The cells were resuspended in 100 µL of fresh YEP medium and used immediately, or stored at -80°C.

Plate-within-a-Plate Protocol

This procedure was based on that described for *Phanerochaete chrysosporium* (Sharma and Kuhad, 2010). Briefly, two Petri dishes, 100 x 15 mm and 60 x 15 mm were placed in concentric position where the inner Petri dish contained 2% malt extract agar (MEA) media while the outer dish contained 2% yeast extract agar (YEA) media with 50 µg/ml concentration of kanamycin (Figure 3). The plates were poured to the height of the inner plate so the media surfaces where flush with each other. The inner MEA plate was inoculated with a fungal disc. The outermost plate was sealed with gauze tape and placed in an incubator at 28°C.

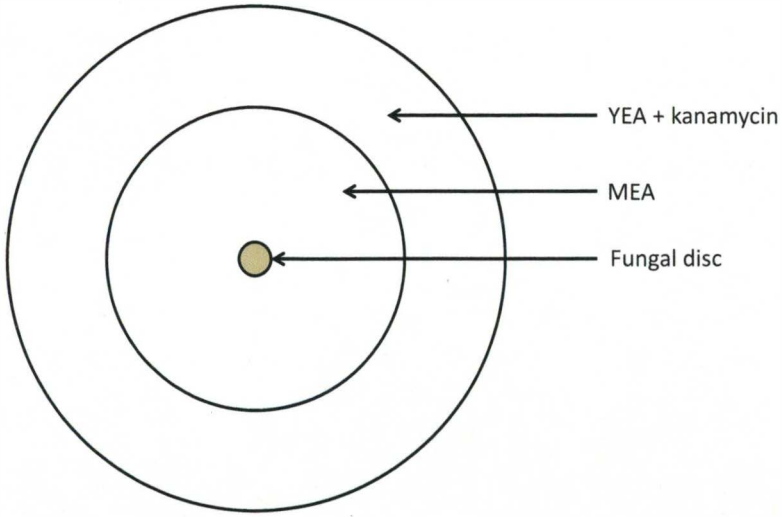

Figure 3: Plate-within-a-plate configuration

Agrobacterium culture containing pGFP (conferring kanamycin resistance) was spread on the outer YEA plate. Any fungal growth that spread to the outer antibiotic ring was removed and transferred to fresh MEA plates containing 200 μg/ml hygromycin (transformed fungal mycelia should be hygromycin resistant; Figure 4). The plates were sealed with gauze tape and were placed in a 28°C incubator. New fungal growth was inspected under a microscope equipped with a UV filter (described below).

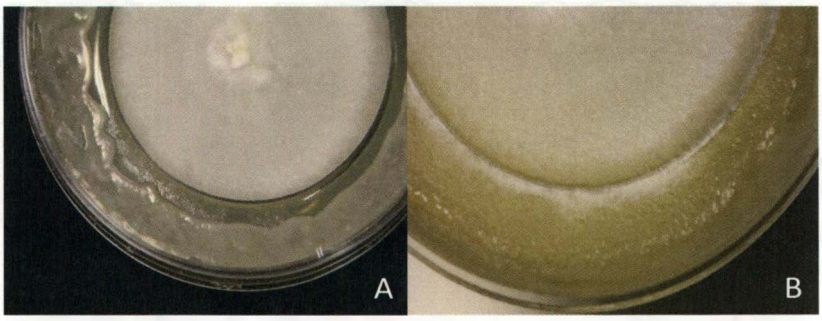

Figure 4: Plate-within-a-plate method showing new *Agrobacterium* growth on the outside plate and *Trametes* growth on the inside plate (A), and after several days of co-incubation (B).

Membrane Protocol

This procedure was based on that described for *Laccaria bicolor* (Kemppainen and Pardo, 2011). Cellophane membranes were cut into 2.5 x 2.5 cm squares and treated with 0.350 g/l EDTA. The membranes were boiled for 10 min, rinsed multiple times with deionized water, and then autoclaved for 1 h at 121°C to remove any impurities. The sterilized membranes were placed on fresh Pachlewski P5 agar plates (Appendix 1). With the use of a laminar hood, a 0.5 cm diameter sample of *T. versicolor* was placed in the center of the membrane. The fungal plates were sealed with gauze tape and were placed in an incubator at 25°C for a three day growth period.

Agrobacterium containing pGFP were grown in YEP with 50 µg/mL of kanamycin at 28°C and 200 rpm overnight. A 15 ml mixture of MIN-medium (Appendix 2), 100 µg/ml kanamycin, and 150 µl of prepared *Agrobacterium* were combined and cultivated for a 24 h period at 28°C and 200 rpm. The optical density was measured at 600 nm, with the optimal absorbance measuring 0.2-0.3. The culture was pelleted at 5,000 rpm for 10 min at 4°C. The supernatant was discarded and the bacteria were resuspended in 15 ml of induction (IND) medium (Appendix 3) supplemented with 100 µg/ml kanamycin.

After the three day growth period of *Trametes* on the cellophane membranes, or until the fungal lawn reached the edges of the membrane, the *Agrobacterium* mixture (OD_{600}= 0.4-0.5) was applied (20-30 µl) with the use of a pipettor to the perimeter of the fungal growth. After the formation of a full fungal lawn (up to one week), the membrane was transferred to modified P0.2% agar (Appendix 4) with 300 µg/mL of hygromycin (for fungal transformant selection) and 500 µg/mL of cefotaxime (to kill remaining *Agrobacterium*). The new plates were placed in an

incubator for the development of new mycelial growth. Any subsequent growth on the antibiotic plate was sampled and observed under UV light as described below.

Optimization of Hygromycin

To determine the optimal hygromycin concentration for *T. versicolor* growth, a serial dilution was constructed. A series of P5 plates (Appendix 1) were prepared containing 0, 50, 100, 150, 200, 250, 300, 350, 400 or 450 μg/mL of hygromycin each. The plates were inoculated with a fungal disc and were placed in a 28°C incubator for growth to develop. After 5 days the fungal growth was assessed.

Microscopy

Any mycelial growth on antibiotic media was collected and viewed under a microscope using simple wet mounts on glass slides. Specimens were visualized using an Olympus BX50 microscope equipped with a UV filter. Images were taken of the mycelia with and without UV light using Viewfinder 3.0.1 software.

Results

The plate-within-a-plate technique that was previously used with *Phanerochaete chrysosporium* (Sharma and Kuhad, 2010) led to many procedural difficulties. For instance, this method was prone to fungal contamination, the Agrobacterium growth was difficult to control, and the preparation was cumbersome – particularly with evenly pouring the concentric plates. Furthermore, this protocol yielded no detectable mycelial transformants.

The membrane protocol previously used with *Laccaria bicolor* (Kemppainen and Pardo, 2011) had an easier preparation compared to the plate-within-a-plate protocol. However, this technique needed to be optimized for the ideal concentration of hygromycin to prevent non-transformed *T. versicolor* growth because the original protocol was specific for *Laccaria.* Optimal hygromycin concentration to prevent non-transformed growth was found to be 150 µg/ml for *T. versicolor* (Figure 5) rather than the original 300 µg/mL for *Laccaria bicolor.*

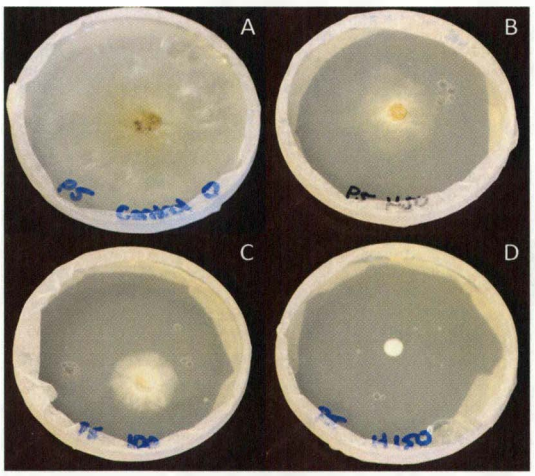

Figure 5: Optimization of hygromycin concentration for *Trametes versicolor* to be used with the membrane protocol. Concentrations were 0 (A), 50 (B), 100 (C) and 150 µg/mL (D). No mycelial growth can be seen on the 150 µg/mL plate.

The membrane protocol was more successful in yielding mycelia transformants than the plate-within-a-plate protocol. Viable transformants from the membrane protocol were detected under UV light confirming the presence of GFP. Transformants emitted blue light (~490 nm) under the UV filter (Figure 6) rather than the typical green expected from green fluorescent protein (~500-508 nm).

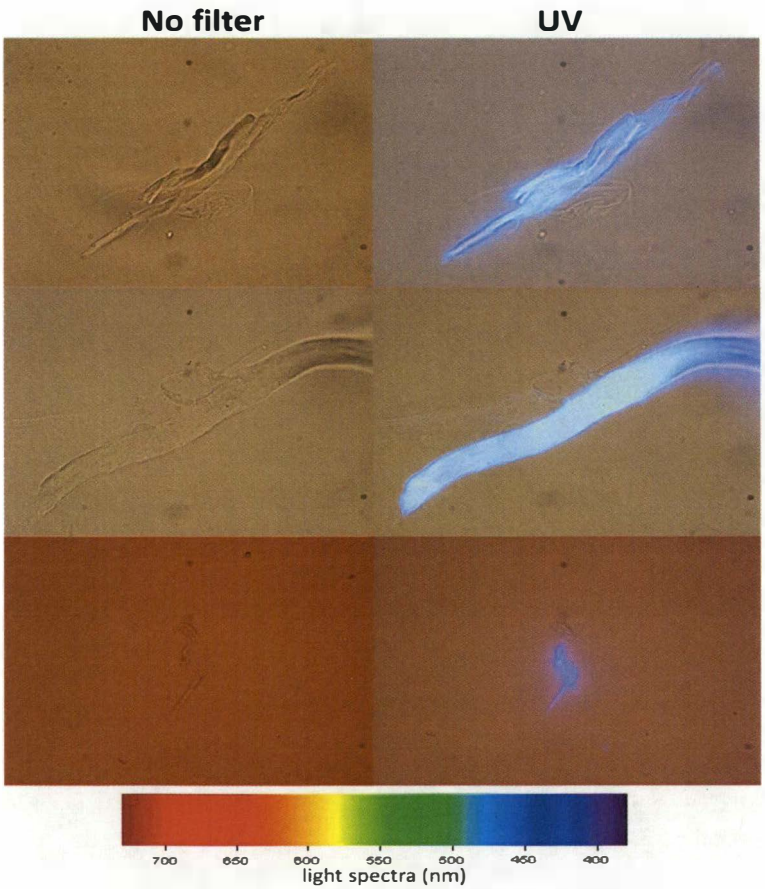

Figure 6: The presence of mycelia containing GFP was detected under UV light using a microscope (1000X). The panels on the left are images with no filter, while the images on the right are those visualized with a UV filter.

Discussion

The purpose of this experiment was to determine the optimal protocol to transform *Trametes versicolor*, a white-rot fungus, with pGFP. The experiment utilized two *Agrobacterium* techniques adapted from the Kemppainen and Pardo (2011) assay with *Laccaria bicolor* and the Sharma and Kuhad (2010) work with *Phanerochaete chrysosporium.*

The Cauliflower Mosaic Virus 35S promoter is typically used in plant transformation protocols for which it is specifically designed (Sharma and Kuhad, 2010; Canam et al., 2011; Tsai et al., 2012). Transformation using a CaMV 35S-driven plasmid to incorporate GFP marker into filamentous fungi is a relatively new idea, with only a few references in the literature (*e.g.,* Kemppainen and Pardo, 2011). This work is the first to show that the plant-based promoter, cauliflower mosaic virus (CaMV) 35S, is effective for expression of any gene in *T. versicolor,* a white-rot fungus.

Agrobacterium tumefaciens-based transformations account for nearly 80% of the transgenic plants and has proved to be a vital asset to biological engineering (Sharma and Kuhad, 2010). It was thought that *Agrobacterium*-mediated transformations would be limited to dicotyledons, gymnosperms, and few moncotyledenous species found in their natural host range. *Agrobacterium*-mediated transformation has been used for recalcitrant species and non-plant species, such as filamentous fungi (de Groot et al., 1998; Sharma and Kuhad, 2010). Transforming *T. versicolor* in this manner is a novel process, with a more traditional protoplast transformation procedure shown to be effective, although difficult (Addleman and Archibald, 1993). Based on the results of this research, the membrane assay designed by Kemppanien and Pardo (2011) is the recommended procedure for transforming *T. versicolor*, because of its ease and relatively high number of transformants.

An unexpected finding during this research project was that the mycelia that were transformed with GFP fluoresced blue under UV light rather than the expected green fluorescence. This could be caused by the CaMV 35S promoter being a plant-based promoter that changed the expression when incorporated into a fungal genome. An alternative explanation is that the cellular conditions (*e.g.,* pH, ionic strength) of the fungal cells change the protein conformation just enough to cause a slight variation in the wavelength emitted. The GFP gene used in these experiments (*gfp5*) and other variants have been modified from the original GFP gene from *Aequorea victoria* to optimize fluorescence in plant systems (Davis and Vierstra, 1998), and similar modifications have been made for other host systems as well (Yang et al., 1996). It is likely that similar modifications will be necessary to ensure green fluorescence, if desired, in future fungal experiments. Nevertheless, the mycelia did fluoresce under UV light and grew on media containing hygromycin, which indicates successful incorporation and expression of the GFP gene.

In the future, the membrane protocol could be used for the production of novel *Trametes* strains for biological pretreatment of biomass feedstocks, specifically for bio-delignification. Previous studies have successfully demonstrated delignification with a mutant *Trametes* strain (m4D) and with the wild-type strain (52J). Both strains were capable of extensive lignin degradation in canola straw, with m4D leaving more sugars for the downstream production of bioethanol (Canam et al., 2011). Therefore, *T. versicolor* is a possible natural and economical pretreatment for the future exploitation of bioethanol from biofuel feedstocks, such as poplar, switchgrass, and *Miscanthus* (Canam et al., 2013).

Conclusions

(1) The membrane assay designed by Kemppainen and Pardo (2011) is the preferred method of *Agrobacterium*-mediated transformation of *Trametes versicolor* based on its ease and its ability to yield viable transformants with pGFP.

(2) The GFP gene used in this experiment (*gfp5*) needs to be optimized specifically for *Trametes versicolor*.

(3) Optimal hygromycin concentration to prevent non-transformed growth of *Trametes versicolor* was found to be 150 μg/ml on P5 medium.

(4) This work is the first to show that the plant-based promoter, cauliflower mosaic virus (CaMV) 35S, is effective for expression in *T. versicolor*, a white-rot fungus.

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Appendix 1: Pachlewski P5 agar medium

Compound	Per Liter
di-NH_4 - tartrate	0.5 g
KH_2PO_4	1 g
$MgSO_4$ x $7H_20$	0.5 g
Maltose	5 g
Glucose	20 g
Thiamine	0.1 mg
$MnSO_4$ x $4H_2O$	5 mg
H_3BO_3	8.5 mg
$(NH_4)_6 Mo_7O_{24}$ x 4 H_2O	0.3 mg
$FeCl_3$	6 mg
$CuSO_4$ x $5H_2O$	0.6 mg
$ZnSO_4$ x $7H_20$	2.7 mg

pH was adjusted to 5.5 with l M KOH, media was sterilized by autoclaving 15 min at 121°C

Appendix 2: MlN-medium

Compound	Per liter
K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2SO_4$	1 g
Na-citrate x $2H_2O$	0.5 g
pH ~7 and is autoclaved	
1M $MgSO_4$	0.8 ml
1% thiamine-HCL, sterilized	0.1 ml
20% glucose, sterilized	10 ml

Appendix 3: IND-medium

Compound	Per Liter
K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(NH_4)_2\ SO_4$	1 g
Na-citrate x $2H_20$	0.5 g
Glycerol	5 g
MES	8.53 g
pH adjusted to 5.3 with (1/10) HCl and is autoclaved	
1 M $MgSO_4$	0.8 ml
1% Thiamine-HCl, sterilized	0.1 ml
20% Glucose	10 ml
Acetosyringone	200 µM

Appendix 4: P0.2% agar medium

Compound	Per Liter
di-NH_4-tartrate	0.5 g
KH_2PO_4	1 g
$MgSO_4$ x $7H_20$	0.5 g
Glucose	2 g
Gycerol	5 g
MES	8.53 g
Agar	20 g
Thiamine *	0.1 mg
Acetosyringone**	200 µM
MnSO4 x 4H2O	5 mg
H_3BO_3	8.5 mg
(NH4)6 Mo_7O_{24} x $4H_2O$	0.3 mg
$FeCl_3$	6 mg
$CuSO_4$ x $5H_2O$	0.6 mg
$ZnSO_4$ x $7H_20$	2.7 mg

*Thiamine: 1ml of filtered sterilized 1,000x stock solution stored at 4°C

**Acetosyringone: added from a 100x stock, added after autoclaving